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Osmo-stress tolerance of tobacco expressing *NtC7*

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Research area:

Environmental Stress and Adaptation to Stress Development

Osmotic Stress Tolerance of Transgenic Tobacco Expressing a Gene Encoding a Membrane Located Receptor-Like Protein from Tobacco Plants[1]

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(Foot notes)

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(Abstract)

Tobacco genes regulated during the early stage of responses to wounding were screened by a modified fluorescence differential display method. Among 28 genes initially identified, a particular clone designated *NtC7* was subjected to further analysis. Its transcripts were found to accumulate rapidly and transiently within 1 h upon treatments with not only wounding but also salt and osmotic stresses. However, jasmonic and abscisic acids and ethylene did not effectively induce *NtC7* transcripts. Amino acid sequence analysis suggested NtC7 to be a new type of transmembrane protein, that belongs to the receptor-like protein family, and a membrane location was confirmed in onion epidermis cells transiently expressing an NtC7-GFP fusion protein. Seeds of transgenic tobacco over-expressing *NtC7* normally germinated and grew in the presence of 500 mM mannitol, but not in the presence of 220 mM sodium chloride or 60 mM lithium chloride. Cuttings of mature transgenic leaf exhibited a marked tolerance upon treatment with 500 mM mannitol for 12 h, at which concentration wild-type counterparts were seriously damaged. These results suggested that NtC7 predominantly functions in maintenance of osmotic adjustment independently of ion homeostasis.

(Introduction)

Plants are continuously exposed to biotic and abiotic stresses that endanger their survival. Among abiotic stresses, water stress is one of the most severe, caused by drought, salt loading and chilling. In order to cope with these stresses, plants have developed various systems such as production of osmolites for osmotic adjustment, synthesis of Na^+/H^+ antiporters for ion sequestration and many others (Bohnert et al., 1995). The operation of these systems usually requires three steps: osmotic stress recognition, signal transduction and production of components for the physiological response. Knowledge on the first and second steps in plants remains relatively limited, and is mostly available from experiments with bacteria and yeast.

The first step is mainly mediated through the osmo-sensor, which recognizes change in osmotic pressure. In *E. coli* and yeast, osmotic stress is detected by the osmo-sensors, EnvZ and SLN1, respectively (Mizuno, 1998, Maeda et al., 1994). A similar protein, AtHK1, has been found in *Arabidopsis* (Urao et al., 1999), although its function *in planta* awaits determination. All have been identified as transmembrane two component histidine kinases. In yeast, another type of sensor, SHO1, has also been detected, which is a transmembrane protein equipped with an SH3 domain (Maeda et al., 1995). Some of these sensor proteins form homo-dimers, the conformation easily changing upon mechanical stimuli to the membrane (Yaku and Mizuno, 1997; Tao et al., 2002). Such conformational alteration is considered to relay the signal into the cell

interior (Posas et al., 1996; Lohrmann and Harter, 2002).

The second step so far identified is the MAP kinase phosphorylation cascade (Wurgler-Murphy and Saito, 1997). In yeast, osmotic signals perceived by the two osmo-sensors, SLN1 and SHO1, is transduced to a MAP kinase (HOG1) through MAPKK (PSB2) (Reiser et al., 2000). HOG1 ultimately activates the synthesis of glycerol to serve as the compatible solute (Albertyn et al., 1994). Whether or not a similar phosphorylation cascade functions in the osmo-signaling pathway in plants is currently not clear. In contrast, the third step has been relatively well studied in plants, and a number of genes have been identified, and characterized as osmotic stress regulated (OR) (Bohnert et al., 1995). The late embryogenesis abundant (LEA) proteins are examples, being known to respond to and reduce the effects of osmotic and cold stresses (Thomashow, 1998). Many other genes encoding proteins involved in osmolite biosynthesis, transporters and regulatory functions have also been isolated (Zhu et al., 1997).

In the present study, we initially screened genes involved in very early stage responses to wounding, and identified a particular gene encoding a membrane-located receptor-like protein, NtC7. Here we report that NtC7 plays important roles in the early response to osmotic stress in tobacco plants.

RESULTS

Identification of *NtC7*

Screening for wound-responsive genes by fluorescence differential display, we initially identified 28 cDNA fragments that were found to change their levels within 3 h after wound treatment (data not shown)(Hara et al., 2000). Among them, a particular clone whose transcripts were rapidly induced after wounding was subjected to a preliminary northern analysis. Total RNA samples were isolated from leaf discs 0, 15, 45, 90 and 180 min after wound treatment, and hybridization was performed using a 337 bp fragment amplified with PCR as the probe. The transcripts of this clone were found to begin to accumulate as early as within 10 min, reaching a maximum level at 1 h, and then to decline to the initial level after 3 h (Fig. 1). Because of such an early transient response, the clone, designated as *NtC7* (*Nicotiana tabacum* C7), was further characterized in the present study.

Sequence Properties and Genomic Organization

A 1210 bp cDNA of *NtC7* was isolated from a cDNA library constructed from mRNAs isolated from wound-stressed leaves (accession no. AB087235)(Fig. 2A). Northern hybridization using this fragment as the probe showed the size of the corresponding transcript to be approximately 1.2 kb, indicating that the cDNA obtained was nearly full length (Fig. 2B). Southern hybridization analysis indicated *NtC7* to

NtC7 to hybridize to a discrete single fragment after digestion of genomic DNA with various restriction enzymes (Fig. 2C). Since *N. tabacum* used in this assay is amphidiploid, the results suggest that a single copy of *NtC7* originated from one of the ancestral parents, either *N. sylvestris* or *N. tomentosiformis*.

Characterization of NtC7 protein

The protein encoded by *NtC7* cDNA was predicted to consist of 308 amino acid residues with a relative molecular mass of about 33.9 kDa (Fig. 2A). A hydropathy plot (Kyte and Doolittle, 1982) indicated the NtC7 polypeptide to possess hydrophobic regions at both N-terminal (amino acids 1~23) and C-terminal (amino acids 275~291) ends (Fig. 3A). Analysis using the PSORT, a computer program for the prediction of protein localization sites in cells (Nakai and Horton, 1999) indicated that the N-terminal region is likely to serve as a signal peptide, cleaved at amino acid positions between 23 and 24 (A/Q) (Fig. 2A). The C-terminal region was predicted to function as a transmembrane domain (Fig. 2A). A BLAST search (Altschul et al., 1990) showed the NtC7 protein to have similarities with rice LRK10 homologs (Feuillet and Keller, 1999) in the N-terminal region (amino acids 20~147)(Fig. 3A and B). LRK10 is a product of leaf rust disease resistance genes originally found in wheat (Feuillet et al., 1997). A distinct feature of NtC7, however, is the lack of a kinase domain, which is present in LRK10 homologs (Fig. 3B and C). In this context, NtC7 rather structurally

resembled tomato Cf-9, a receptor for avr-9 of *Cladosporium fulvum* (Jones et al., 1996)(Fig. 3C).

Cellular Localization

To identify the cellular localization, a reporter gene encoding green fluorescence protein (GFP) was fused to *NtC7*, and subjected to transient assay using onion epidermis cells (Fig. 4). After biolistic bombardment, individual cells were observed for localization of NtC7 by GFP fluorescence (Fig. 4A-C), using 4',6 diamidino-2-phenylindole (DAPI) staining for nuclei (Fig. 4D-F), and interference contrast images for whole cell structures (Fig. 4G-I). CaMV 35S::GFP control construct (psGFP(S65T) showed GFP signals in both cytoplasm and nucleus (Fig. 4A). The CaMV 35S::NtC7-GFP (pNtC7-GFP) showed GFP signals predominantly at the membrane (Fig. 4B). This pattern was identical with that of a positive control, CaMV 35S::IMK3-GFP (pIMK3-GFP), a plamid containing a cDNA for inflorescence meristem receptor-like kinase 3 (IMK3) of *Arabidopsis* (Takemura et al., 2000), showing GFP signal at the membrane (Fig. 4C). The results suggested NtC7 to be a membrane-located protein.

Expression Analysis

Transcript accumulation of *NtC7* was analyzed in leaves subjected to abiotic stress conditions (Fig. 5). Since *NtC7* was primarily identified from leaf discs floated on

water, the stress was possibly from wounding, osmotic changes or a combination of the two. To distinguish these, a healthy leaf of an intact plant was cut with a pair of scissors, and RNA was extracted from both wounded and adjacent unwounded leaves. Northern hybridization indicated simple injury to be sufficiently effective to locally and systemically induce *NtC7* transcripts (Fig. 5A). Osmotic stress was achieved by 500 mM mannitol treatment to leaves which were detached and left to absorb water for 4 h prior to further processing to depress initial wound effects. Transcripts temporarily accumulated by 45 min after osmotic shock, but were diminished 4 h later (Fig. 5B). Control leaves, which were kept in water for the same period, transcript accumulation was not induced (Fig. 5B). Treatment with 200 mM NaCl also induced *NtC7* transcripts showing a similar accumulation pattern as that for osmotic stress (Fig. 5C). Since wound and osmotic signals are often transmitted through jasmonic acid (JA), abscisic acid (ABA) and ethylene respectively, detached leaves were also treated with these chemicals and transcript induction was estimated (Fig. 6). In order to confirm treatment efficacy, samples were hybridized with cDNAs for ornithine decarboxylase (*ODC*) that responds to JA, *DIN1* that responds to ABA (Hara et al., 2000) and *basic PR-1* that responds to ethephon (Hiraga et al., 2000). While all chemicals correctly induced the marker transcripts, neither of them induced *NtC7* transcript accumulation (Fig. 6). These observations indicate *NtC7* to respond to both wounding and osmotic stresses independently of JA, ABA and ethylene.

Physiological Assay of Transgenic Seedlings

In order to examine the physiological function of NtC7, transgenic tobacco plants constitutively expressing *NtC7* were constructed. More than 10 transgenic lines were produced, and after confirmation of integration and expression of *NtC7* by PCR and northern analyses (Fig. 7A), five lines were selected for further examination. Seeds of the T_3 generation of the line #1 were sown on agar plates containing half strength Murashige-Skoog (MS) medium supplemented with or without appropriate concentrations of mannitol, NaCl, or LiCl, and germination and growth examined. The transgenic seedlings showed clear resistance to osmotic stress caused by mannitol at as high as 500 mM, at which concentration the growth of control wild-type seedlings was completely suppressed (Fig. 7B). In contrast, their growth was totally retarded in the presence of 220 mM sodium chloride, suggesting the plants to be susceptible to Na^+ ions (Fig. 7B). This was confirmed by their sensitivity to 60 mM lithium chloride, at which concentration osmotic status of cells is not seriously affected, the toxicity of Li^+ being even higher than that of Na^+ ions (Fig. 7B). The same results were obtained with other transgenic lines (#6, #14, #21 and #53) showing resistance to mannitol and susceptibility to salt ions (data not shown).

Physiological Assay of Transgenic Mature Leaves

To determine whether or not mature plants also exhibited the tolerance, stress conditions were first determined. Healthy leaves of wild-type plants were cut out at the petiole, and treated with 500 mM mannitol solution by absorption for appropriate time period. Leaves were then transferred to water and allowed to recover from wilting (Fig. 8A). Results showed that, by as short as 2 h treatment, leaves were already unable to recover from wilting, showing necrotic spots on the surface (Fig. 8A). Upon treatment for 12 h, leaf exhibited severe necrosis all over the surface, and ultimately died after 2 days. Based on these observations, transgenic leaves were assayed for recovery from wilting after 12 h treatment (Fig. 8B). The same symptom as the control wild-type plants was observed with transgenic lines #9 and #40, which did not express the transgene (Fig. 7A). These leaves also died after 2 days. In contrast, transgenic lines #1, #6 and #53, actively expressing *NtC7*, rapidly recovered, showing apparently the same feature as untreated samples (Fig. 8B). These results were consistent with those of seedlings (Fig. 7B), and strongly suggested that *NtC7* played an important role in tobacco response to osmotic stress.

DISCUSSION

This paper describes isolation and properties of a gene encoding a receptor-like membrane protein, that functions in response to osmotic stress. The predicted NtC7

protein has a hydrophobic signal sequence at the N-terminus (amino acid positions 1~23), a helix transmembrane region (amino acid positions 275~291), and a hydrophilic region at the C-terminus (amino acid positions 292~308)(Fig. 2A and Fig. 3A). A homology search with the predicted amino acid sequence indicated that NtC7 resembles the receptor domain of receptor-like kinases (RLKs). Plant RLKs are grouped into 4 types depending on amino acid sequences. Type-1 constitutes so-called leucine-rich repeat (LRR) proteins having 24 amino acid repeat units containing many leucines, type-2 proteins have homology to the S-locus glycoprotein (SLG), type-3 proteins have lectin-like domain which are thought to bind oligosaccharides, and the type-4 group demonstrates homology to epidermal growth factor repeated sequences (Hardie, 1999). The amino acid sequence of the NtC7 protein at the proximal N-terminus showed highest similarity to the proximal N-terminus of the receptor domain in rice homologs of wheat LRK10 (leaf rust disease resistant kinase), belonging to the type-2 group (Feuillet et al., 1997)(Fig. 3B and C). In contrast, the distal N-terminus of the predicted NtC7 protein did not show any similarity to these proteins. Considering these structural properties, we conclude that NtC7 belongs to a new subfamily of RLKs. Since the NtC7-GFP fusion protein was shown to localize in the membrane fraction, it is highly probable that it is a membrane associated receptor-like protein with the C terminus oriented to the cytoplasm.

In order to identify the physiological role of NtC7, transgenic tobacco plants were

constructed and analyzed for their stress responses. Mature leaves of transgenic plants showed a tolerance to osmotic stress, as clearly seen by rapid recovery from severe wilting caused by 500 mM mannitol, at which concentration, control leaves suffered serious damages such as necrosis. Transgenic seedlings were also highly tolerant to the same stress. A notable finding, however, was that they were susceptible to salt stress, showing a similar sensitive response as the wild-type control. The simplest explanation for this is that transgenic plants produced some compatible solutes, which confer tolerance to osmotic stress, but not to sodium ion toxicity. Since major compatible solutes in tobacco plants are reported to be derivatives of sugars and amino acids like proline (Yoshiba et al., 1997), it is conceivable that over-expressed NtC7 activates the production of such compounds. Judging from its structure, however, it is unlikely that NtC7 directly participates in their synthesis. Instead, it may be involved in the signaling pathway to activate osmotic stress responsive genes, functioning, for example, as part of the osmo-sensor system.

The best studied osmo-sensors are two-component histidine kinases, identified in *E. coli*, budding yeast and *Arabidopsis* (Cai and Inouye, 2002; Li et al., 2002; Hwang et al., 2002). They are suggested to form homo-dimers, whose conformation is sensitive to changes in membrane architecture (Wurgler-Murphy and Saito, 1997; Yaku and Mizuno, 1997). Structurally, however, NtC7 distinctly differs from any known osmo-sensors, but resembles RLKs. Although it is unclear whether RLKs form dimers,

Arabidopsis RLK5 has been proposed to form a homo-dimer through its leucine-rich regions and to interact with a kinase-associated protein phosphatase (KAPP)(Braun and Walker 1996). By analogy, it may be possible that NtC7 forms dimers through the leucine-rich region near the C-terminus (Fig. 2A). Another specific feature of NtC7 is the lack of a kinase domain, thus structurally resembling tomato Cf-9 (Fig. 3C), a transmembrane protein that confers resistance to tomato leaf mold and is considered to transmit the pathogen signal to the cytoplasmic protein through its cytoplasmic tail. One such cytoplasmic protein was proposed to be a protein kinase, typically represented by Pto (Hammond-Kosack and Jones, 1997)(Fig. 3C), a cytoplasmic serine/threonine kinase considered to play a critical role in several pathogen signaling pathways (Hammond-Kosack and Jones 1997; Braun and Walker 1996). Indeed, proteins like Cf-9 and Pto have been repeatedly suggested to interact with each other in an analogous way with counterparts in the mammalian immune system (Hammond-Kosack and Jones 1997; Braun and Walker 1996). Taking account of structural similarities, it is conceivable that NtC7 interacts with partner protein(s) through its C-terminal tail region, thereby transmitting osmo-signals to cytoplasmic components.

Transcripts of *NtC7* spatially and transiently accumulated upon osmotic stress. Since a low level of transcripts was here constitutively observed even in the absence of stress, such a rapid induction may indicate that a relatively large amount of NtC7 is only needed upon stress. A similar pattern of transcript induction is observed for genes

encoding, for example, two-component signaling component ARR for cytokinine response (Kiba et al., 1999), RLKs for pathogen recognition (Du and Chen, 2000), MAP kinase (WIPK) in the wound response (Yap et al., 2002) and WRKY transcription factor (TIZZ) in the hypersensitive response (Yoda et al, 2002). All are involved in cellular signaling pathways, supporting the idea that NtC7 also functions in osmo-signal transduction. Although more detailed analyses of the protein level are necessary, the temporal expression of these genes suggests that one of the mechanisms for activation and desensitization is strict control at the transcriptional level. Perhaps plants respond to environmental cues by rapid production and degradation of relevant proteins only as necessary, thereby best coping with severe biotic and abiotic stresses.

The present findings further suggest that one wound signals could be associated with osmotic change. To date, many signal molecules that induce transcription of so-called wound-responsive genes have been identified, including JA, ABA, ethylene, small peptides, oligosaccharides and reactive oxygen (Kessler and Baldwin, 2002). In addition, physical signals such as hydraulic pressure, electric currents (Leon et al., 2001) and pH change (Hara et al., 2000) have also been suggested to play a role. The induction profile of NtC7 transcripts, featuring rapid accumulation in both local and systemic leaves and independent of JA, ABA and ethylene supports the idea that the hydraulic status is one of the factors underlying wound signaling.

Salt and drought tolerance is one of the most important traits for crops, as world

arable lands are continuously being injured from salt accumulation and desiccation. Transgenic technology has been expected to be helpful to solve such problems. Since salt induces both ion toxicity and osmotic stress, introduction of multiple genes that cope with these stresses would be practical. In this context, our *NtC7* may be useful if utilized in the combination with genes involved in salt ion homeostasis, such as *HKT* that encodes a Na^+/K^+ synporter (Maser et al., 2002).

MATERIALS AND METHODS

Plant Materials and Wound and Chemical Treatments

Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown in soil in a growth cabinet at 23°C under a 14/10 hr light/dark photocycle. Wound stress was applied by cutting mature leaves with a pair of scissors. Wounded (local) and adjacent upper unwounded (systemic) leaves were harvested at appropriate time points. After put in water for 4 h to diminish cutting stress, samples were transferred into a solution containing one of the following chemicals: 200 mM NaCl, 60 mM LiCl, 500 mM mannitol or 100 μM ABA. For treatment with volatile chemicals, samples were exposed to 50 μM jasmonic acid methyl ester (MeJA) or 100 μM ethephon (ethylene) in a sealed box.

Fluorescence Differential Display

The fluorescence differential display (FDD) and co-migration tests were essentially performed as described earlier (Hara et al., 2000). Briefly, total RNAs were isolated from treated or untreated sample leaves, digested with Dnase I, and cDNAs were synthesized and subjected to PCR using rhodamine-labeled 3'-anchored primers (Takara) and 12-mer arbitrary primers. The reactions were carried out with 25 cycles of 94°C for 30 sec, 40°C for 1 min, 72°C for 1 min, and 72 °C for 5 min for a final extension. After PCR amplification, samples were fractionated by 5% denaturing polyacrylamide gel electrophoresis, and migration patterns were analyzed with an image analyzer (FM-BIO Takara). cDNAs differentially amplified were eluted, reamplified by PCR with the same pair of primers as used for the first amplification and subcloned into the pT7blue vector (Novagene).

cDNA Library Construction and Screening

Total RNA was isolated from wound-treated leaves tobacco by the AGPC method (Chomczynski and Sacchi, 1987) with a slight modification, and used for cDNA library construction with the λZapII vector (Stratagene). Briefly, cDNAs were ligated to the vector at *Eco*RI and *Xho*I sites. After transformation, the library was screened with a ^{32}P-labeled *NtC7* fragment obtained by FDD, and positive clones were rescued in the pBluescript SK-phagemid vector by in vivo excision. After amplification in *E. coli*

coli JM109, nucleotide sequences were determined by the dideoxynucleotide chain termination method (ABI PRISM BigDye Terminator). Sequence editing, prediction of amino acid sequences, hydropathy plots and multiple alignment, as well as similarity and localization searches, were carried out with appropriate computer software (GeneWorks, National Center for Biotechnology Information and PSORT server).

DNA and RNA Gel Blot Analysis

Genomic DNA was isolated from green leaves by the CTAB method (Murray and Thompson, 1980) with a slight modification, and 20 μg aliquots were digested with one of the restriction enzymes *Bam*HI, *Eco*RI, *Hnid*III or *Xho*I, separated by electrophoresis on 0.6% agarose gels and transferred onto nylon membranes (Hybond-N+, Amersham). After crosslinking using a UV crosslinker (RPN 2501, Amersham), the membranes were subjected to hybridization with appropriate ^{32}P-labeled probes at 65°C for 16 h. After successive washing with 0.1 x SSC at 65°C, they were used to expose either BAS (Fuji film) or x-ray film (Kodak). Total RNAs were isolated by the ATA method (Gonzalez et al., 1980) with a slight modification, and used for RNA gel blot analysis. A 20 μg aliquot per lane was fractionated by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N+, Amersham). Hybridization was performed in the same way as for the DNA hybridization described above except hybridizing temperature being 42 °C.

Plasmid Construction and Histochemical Analysis

The coding region of NtC7, with the stop codon deleted, was amplified by PCR with *Ex*Taq DNA polymerase (Takara) using forward (5'-GTCGACATGTTGACAAGAGGGCTGC-3') and reverse (5'-CCATGGAACAGTTCTGTTCATCGGAGG-3') primers containing a *Sal*I site upstream and a *Nco*I site downstream of the deleted stop codon, respectively. This PCR fragment was first introduced into the pGEM-Teasy (Promega) vector for amplification in the *E. coli* strain JM109. The plasmid was digested with *Sal*I and *Nco*I, and the resulting fragment was introduced into the *Sal*I/*Nco*I site of the psGFP(S65T) vector by fusing the coding region in-frame to that of GFP. Particle bombardment was performed according to the manufacturer's instructions (PDS-1000, Bio-RAD), with 10 μm diameter gold particles coated with plasmids. A 3-cm square onion scaly leaf fragment was placed under the stopping screen at a distance of 6 cm or 9 cm and bombarded twice per sample in a vacuum of 28 inches of mercury using a helium pressure of 1100 psi to accelerate the macrocarrier. Bombarded leaves were kept in the dark for 12 h at 25°C before analysis. DAPI staining and GFP epifluorescence assay were performed essentially as described (Nishiyama et al., 2002).

Plant Transformation

For plant transformation, PCR-amplified *NtC7* using forward (5'-GCTCTAGAGAACATGTTGACAAGAGGGC-3') and reverse (5'-GAGCTCTTAACAGTTCTGTTCATCGG-3') primers containing *Xba*I and *Sac*I sites at the 5' and 3' ends, respectively, was introduced into the pGEM-Teasy vector as described above. Digested fragments were ligated to *Xba*I/*Sac*I sites of pBI121 vector (Clontech), and introduced into the *Agrobacterium* strain LBA4404 cells. Tobacco transformation was performed as described previously (Yap et al., 2002).

Bioassay

Transgenic *NtC7* plants were grown to maturity to yield progeny seeds. For estimation of stress tolerance, approximately 10 seeds of the T_3 were sown on a 1/2-strength MS agar plate containing appropriate concentrations of salt or mannitol and cultured under continuous light at 23°C. After appropriate time periods, germinated seedlings were counted and measured for growth. Healthy, unwounded leaves from wild-type and T_0 transgenic plants were cut out with a sharp razor blade at petioles, and put in a vessel containing 500 mM mannitol. After standing for appropriate time intervals at room temperature under continuous light, samples were transferred to water, allowed to recover from wilting for additional 48 h, and photographed.

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Figure legends

Figure 1. Accumulation of *NtC7* transcripts upon wounding. Healthy leaves were detached and wounded by cutting into pieces with a pair of scissors, and floated on water. The wounded leaves were harvested at the indicated time points. Blots containing 35 μg of RNA per lane were successively subjected to hybridization with the *NtC7* fragment obtained by FDD and actin cDNA.

Figure 2. Analyses of the *NtC7*. A, The nucleic acids are presented on the top line and the derived one-letter amino acid sequence is shown below. The stop codon is indicated by an asterisk. The original *NtC7* clone obtained by FDD screening is underlined. The position of cleaving site of the predicted signal peptide is indicated by a closed triangle. The predicted transmembrane region is indicted by small letters. Leucines and isoleucines near the transmembrane domain are marked with circles. The accession number of the cDNA is AB087235 under the name of NtC7. B, Northern blot hybridization. Total RNA was probed with the full length *NtC7* cDNA, and transcript size was estimated from the migration position of marker RNAs (M) stained with ethidium bromide (EtBr) and signals due to cross-hybridization. C, Southern blot analysis. Genomic DNA (20 μg per lane) was digested with the indicated restriction endonuclease, separated on an agarose gel, blotted and probed with a radioactively labeled *NtC7* fragment.

Figure 3. Properties of NtC7. A, A hydropathy plot of the NtC7 polypeptide. Hydropathy analysis was performed using a window of nine amino acids. A putative signal peptide, a region homologous with LRK10 and a putative transmembrane domain are indicated with arrows. B, Alignment of NtC7 with LRK10 homolog 1 (accession no. AAC27489)(LRK10h1) and LRK10 homolog 2 (accession no. AAC02535)(LRK10h2) from rice was performed with the ClustalW program. Identical residues shared among the three are shaded. C, Pattern diagrams of plant receptor-like proteins. The molecular size of each is shown in numbers of amino acids (aa) on the right side. LRK10h1 is a rice homolog of wheat LRK10 (accession no. T06793), Cf-9 is a tomato receptor for avr-9 of *Cladosporium fulvum* (accession no. AAA65235). For reference, Pto, a protein kinase proposed to interact with Cf-like proteins, is also shown (accession no. A49332). The homologous region between NtC7 and LRK10 homolog 1 (LRK10h1) in the receptor domain, a putative transmembrane domain (TM) and a kinase domain are indicated by hatched, shaded and closed boxes, respectively.

Figure 4. Membrane localization of NtC7 in onion epidermal cells. Onion bulbs were bombarded with gold particles coated with psGFP(S65T) (A, D and G), pNtC7::GFP (B, E and H) or pIRM3::GFP plasmids (C, F and I). The proteins were transiently expressed and individual cells were observed by epifluorescence for GFP (A-C), by

staining with DAPI (D-F) or under interference contrast (G-I). Nuclei identified with DAPI staining and in interference contrast images are indicated by arrows (D-I).

Figure 5. Effects of osmotic and salt stresses on *NtC7* transcript accumulation. Healthy leaves were wounded by cutting with a pair of scissors, and wounded (local) and unwounded adjacent (systemic) leaves were sampled at the indicated time points, and RNAs assayed by northern hybridization with the indicated probes (A). Healthy leaves were detached and put in buffer solution. At 4 h after the first wounding, leaves were transferred to a buffer solution with or without 500 mM mannitol (B) or 200 mM NaCl (C) for the indicated time period, and RNAs were assayed by northern hybridization with the indicated probes.

Figure 6. Effect of phytohormones on *NtC7* transcript accumulation. A leaf cutting was prepared from a healthy plant, left for 4 h for acclimatization to the initial wound stress, then exposed to 50 μM MeJA (A), 100 μM ABA (B) or 100 μM ethephon (C) for the indicated time period. Total RNAs were then subjected to northern assay. Control samples for ABA treatment were treated with water (Water) (B).

Figure 7. Effects of osmotic and salt stresses on transgenic tobacco seedlings expressing *NtC7*. A, Integration and expression of *NtC7* in transgenic lines were

examined by PCR (upper panel; transgenes) and northern hybridization (lower panel; transcripts). B, Germination and growth test. A batch of 10 seeds of transgenic line #1 or wild-type plants were sown on the 1/2 strength MS-agar medium containing 60 mM LiCl, 220 mM NaCl or 500 mM mannitol along a nylon mesh, cultivated for 2 weeks and photographed.

Figure 8. Effects of osmotic stress on tobacco mature leaves. A, Time course analysis of recovery from wilting in wild-type plants. A young, healthy leaf from ~2 month-old plants was cut out, put into a vessel containing 500 mM mannitol, and left at room temperature for indicated time period. B, Tolerance of transgenic lines against osmotic stress. A young, healthy leaf from each of ~2 month-old transgenic plants with indicated numbers was cut out, put into a vessel containing 500 mM mannitol, and left for 12 h at room temperature. Lines #9 and #40 contained the transgene without expression, and lines #1, #6 and #53 actively expressed the *NtC7* (see Figure 7A). After treatments, each sample was transferred to a vessel containing water to recover for 48 h and photographed.

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